Marmin of *Aegle Marmelos* Correa Antagonizes $AChM_3$ Receptors: *In Silico* and *In Vitro* Studies on Isolated-Guinea Pig Illeum Smooth Muscle

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Abstract. A previous study has shown that ethanolic extract of leaves of *A. marmelos* Correa on isolated ileum had an antagonistic effect on the contraction induced by histamine. Apart from H_1 receptors, muscarinic acetylcholine (AChM) receptors are also involved in the mechanism of ileum contraction. Marmin is predicted to antagonize the $AChM_3$ receptors. This study was aimed at determining the effect of marmin on smooth muscle contraction of isolated guinea pig ileum induced by metacholine, an $AChM_3$ agonist. The methods used were *in vitro* (isolated organ) and *in silico* (docking PLANTS) studies. In the *in vitro* study, marmin exhibited a competitive antagonistic effect at a concentration of 100 µM (pA_2 = 1,728) on the $AChM_3$ receptors. A reversibility assay of the $AChM_3$ receptors showed that by washing the ileum with Tyrode's buffer every 6 minutes for 30 minutes, the bond of marmin to the receptors was still not completely detached. In the *in silico* study, marmin was observed to be bound to the $AchM_3$ receptors (docking score: -102.086). The marmin bond was weaker than that of the native ligand of the $AChM_3$ receptors (tiotropium, docking score: -115.107), but stronger than that of the AChM3 receptor agonist and antagonist. Based on the results, we conclude that marmin has competitive antagonist activity on $AChM_3$ receptors.

Keywords: *marmin; isolated guinea pig; PLANTS; ileum; $AChM_3$ receptors; antagonism.*

1 Introduction

Maja (*Aegle marmelos* Correa., Rutaceae Family) are herbs that are used traditionally in South and South East Asian countries. Marmin – also named 7-(6',7'-dihydroxygeranil-oxy) coumarin – is a coumarin group of compounds contained in the roots and stems of *Aegle marmelos* Correa. Maja roots are used

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empirically for the treatment of dysentery, dyspepsia, and chronic diarrhea. The dried roots are used in the treatment of the nervous system, inflammation, vomiting, and rheumatism [1]. In India, Bangladesh, and other countries of South Asia, this plant is used in the treatment of asthma and heart disease [2]. Various medicinal compounds have been isolated from this plant, including lupenon triterpene compounds, stigmasterol, sitosterol, aegeline, skimmianine, marmin, zeorin, dustanin, aureptene and epoxyaureptene [3].

Several pharmacological studies of this plant have been carried out. Ethanolic extract of leaves of *Aegle marmelos* Correa has been reported to inhibit smooth muscle contraction induced by histamine [2]. Potential antiallergy screening of *Aegle marmelos* Correa provided three potential compounds, namely aegeline, skimmianine and marmin [4,5]. Among them, marmin was the most potent and hence it was selected for further study. In addition, marmin exhibited inhibition of mast cell degranulation via inhibition of Ca^{2+} influx. Reportedly, marmin succeeds in decreasing the contraction of the guinea-pig tracheal smooth muscle. Its action is related to antagonism on histamine receptors, inhibition of histamine release from mast cells, inhibition of intracellular Ca^{2+} release from the intracellular store, and inhibition of Ca^{2+} influx on voltage-dependent Ca^{2+} channels [6,7].

Besides H_1 receptors, $AChM_3$ receptors are also involved in the mechanism of ileum contraction [8]. Therefore marmin was predicted to have antagonistic action on this receptor. This study focused on a pharmacodynamic assay of marmin and its interaction with $AChM_3$ receptors. We used two methods to study the interaction between marmin and $AChM_3$ receptors: *in vitro* study (isolated ileum method) and *in silico* study (docking PLANTS). The outcome of this research was expected to produce scientific data that could support the previous study in order to obtain a more complete pharmacodynamic image of marmin.

2 Materials and Methods

2.1 Materials

Marmin was obtained from Prof.Dr. Sugeng Riyanto (Department of Pharmaceutical Chemistry, Faculty of Pharmacy, Gadjah Mada University). Male guinea pigs with a body weight ranging between 400 and 500 grams were obtained from the Department of Pharmacology and Clinical Pharmacy, Faculty of Pharmacy, Universitas Gadjah Mada. All animal handling protocols were performed in accordance with the guidelines of laboratory animal care of the department. The chemicals used in the study were Tyrode's buffer solution, carbogen gas (containing 95% oxygen and 5% carbon dioxide, obtained from

PT. Aneka Gas and Industrial Semarang), metacholine (obtained from Sigma, USA), distilled water (obtained from Instrumental Chemistry Laboratory, Faculty of Pharmacy, University Gadjah Mada).

2.2 In Vitro Study

The first stage of the assay was to test the effect of 100 mL DMSO on ileal smooth muscle contraction induced by metacholine. The purpose of the assay was to ensure that the DMSO used as a marmin solvent would not affect the response of the ileal smooth muscle contraction induced by metacholine. Marmin activity as a $AChM_3$ receptor antagonist was evaluated by observing changes and shifts in the curve of ileal smooth muscle contraction. The contraction was induced by cumulative concentrations of metacholine, ranging from 10^{-8} to 10^{-3}M.

An organ bath was filled with 20.0 mL of Tyrode's buffer solution, then the organ was placed in the organ bath until a steady state equilibrium was reached (30 min). Subsequently, the single concentration of agonist was introduced to the organ bath and the contraction response was recorded (polygraph paper). After the contraction reached a plateau, the organ was washed by Tyrode's buffer for 60 min with replacement of the Krebs solution every 15 min. Subsequently, cumulative concentrations of the agonist ranging from 10^{-8} to 10^{-3} M were added to the organ bath. After maximum contraction, the organ was washed and the procedure was repeated to get two contraction effects of the agonist. After a washing period of 60 min, 10 μM marmin was added to the organ bath at 10 min prior to administration of cumulative concentrations of agonist. After rewashing the organ, this procedure was repeated for each concentration of 100 μM marmin.

A reversibility assay was performed to observe the ability of the organ tissue to return to basal condition after the marmin treatment. The assay was performed to evaluate the reversibility of the interaction between the receptor and its agonist. The assays were performed before and after the marmin activity assay. The ileum was washed briefly for 30 minutes with Tyrode's buffer solution and with replacement every five minutes. After reaching a stable condition of ileum, the organ was contracted by cumulative concentrations of methacoline, after which the contraction response was recorded. The receptor agonist concentration curves before and after treatment with marmin were compared.

2.3 In Vitro Data Analysis

In the *in vitro* study, the research data concerned ileal smooth muscle contraction. The data were transformed into a percentage of the maximum

response achieved by the agonist. Subsequently, the response percentages were plotted against the logarithm of the agonist concentrations.

The EC_{50} values (concentration of agonist that can produce a response of 50% of the maximum response) of receptor agonist in presence and absence of marmin were calculated based on the curve of the response percentages vs. the logarithm of the agonist concentrations. The EC_{50} was calculated based on Equation 1 and then transformed into a pD_2 value (Equation 2). The data were then represented as mean of pD_2 agonist ± standard error ($pD_2 \pm SE$). The pD_2 values were statistically analyzed using the ANOVA test.

$$LogEC_{50} = \left[\frac{50 - Y_1}{Y_2 - Y_1} \times \left(X_2 - X_1 \right) \right] + X_1 \quad (1)$$

where:
X1 : log of concentration with response below 50%
X2 : log of concentration with response above 50%
Y1 : % response below 50%
Y2 : % response above 50%

$$pD_2 = -\log EC50 \quad (2)$$

Marmin was designated as $AChM_3$ receptor antagonist if there was a decrease of the pD_2 value of metacholine due to marmin. The data distribution of the pD_2 values of metacholine was analyzed using a normality test (Kolmogorov-Smirnov method). Subsequently, the shift in pD_2 value was analyzed with parametric statistical methods (ANOVA test followed by LSD test at 95% confidence level).

Determination of antagonist type was performed using a Schild-plot analysis in the form of a regression analysis. The Y axis is the ratio of the EC_{50} of agonist in presence of antagonist to EC_{50} of agonist in absence of antagonist, and then minus one. The X-axis is the logarithm of the concentration of antagonist. The antagonist type is determined based on the value of the slope generated by the Schild-plot equation. If the slope value is close to one, the receptor antagonist is competitive, whereas if the value of the slope is not close to one, it is non-competitive. The pA_2 value (antagonist affinity of marmin to the receptor) is the intercept value of the Schild-plot [7].

2.4 In Silico Study

Protein Preparation. Protein preparation was perfomed using the YASARA program. Protein files (4DAJ.pdb) were downloaded from the website http://www.pdb.org. The 4DAJ.pdb file was loaded into the YASARA program.

The first editing step was to add the hydrogen into the system with the YASARA program, which yielded the 4DAJ.yob file. The second step was to remove the original ligand and take only the target protein with a pocket for simulating docking. The third step was to change 4DAJ.yob to protein.mol2.

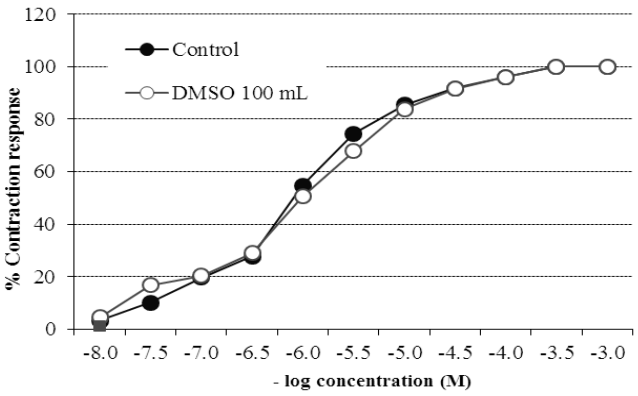
Ref_ligand Preparation. Ref_ligand preparation was performed using the YASARA program. The chemical compounds on the 4DAJ.yob had to be removed, except for the ligand. This file was saved as ref_ligand.mol2.

Docking Simulation using PLANTS. The compounds that were docked with the protein were marmin, tiotropium (native ligand), ipratropium, darifenacine, atropine, and oxybutimine. After the docking process was completed, ten ligand conformations that bind to the 4DAJ with different energies were observed. For evaluation and interpretation of the docking data, the conformations with the lowest score were selected.

3 Results and Discussion

A preliminary assay was carried out to evaluate the effect of DMSO on ileal

(a)



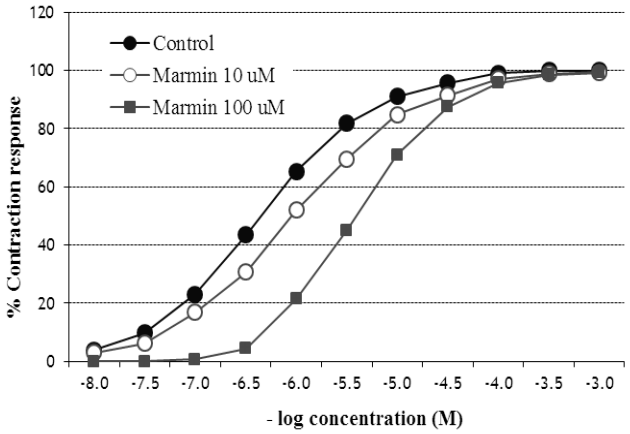
(b)

Groups	pD_2	E_{max} (%)
Control	6.09 ± 0.05	100 ± 0.00
DMSO	6.12 ± 0.04	100 ± 0.00

Figure 1 Effect of DMSO on the response of ileal smooth muscle contraction induced by metacholine. (a) The percentage curve of contraction responses vs. logarithm of the agonist concentrations of metacholine in absence and presence of 100 mL DMSO (n = 5, mean ± SEM). (b) The pD_2 and Emax values of metacholine in absence and presence of 100 mL DMSO (n = 5, mean ± SEM).

smooth muscle contraction induced by methacholine. In the assay, there was no significant effect of DMSO (100 µL) on ileal smooth muscle contraction induced by metacholine (Figure. 1). In the study of the acetylcholine receptors, marmin concentrations of 10 and 100 uM were able to shift the agonist contraction response curve to the right. Marmin also decreased the pD_2 value of metacholine in a concentration-dependent manner. However, the curve shifting due to marmin was not accompanied by a decrease of maximum effect (E_{max}). During the treatment with marmin, the maximum response of isolated ileal smooth muscle contraction could still be achieved in the presence of metacholine at 3 x 10^{-4} M.

(a)



(b)

Groups	pD_2	E_{max} (%)
Control	6.61 ± 0.05	100 ± 0.00
marmin 10 µM	6.00 ± 0.09	100 ± 0.00
marmin 100 µM	5.30 ± 0.05*	100 ± 0.00

* Significant difference ($P < 0.05$) compared to control

Figure 2 (a) Concentration-response curves to metacholine in the absence or presence of marmin at concentrations of 10 and 100 µM in guinea pig ileal smooth muscle (data represent n = 5-10, mean ± SEM). (b) The pD_2 and Emax values of metacholine in absence and presence of marmin at concentrations of 10 and 100 µM (n = 5, mean ± SEM).

The shift of the contraction response curve due to marmin is presented in Figure 2. After pretreatment with 100 uM marmin, contraction responses were not observed until the administration of a high concentration of metacholine (1 x 10^{-6} M). Whereas, after pretreatment with 10 uM marmin, contraction was first observed after treatment with methacholine at 3 x 10^{-8} M. The pD_2 values of metacholine in absence (control) and presence of 10 and 100 uM marmin were

6.39, 6.08, and 5.46, respectively. In comparison to that of the control group, the pD_2 value of metacholine of the 100 uM marmin group differed significantly ($p < 0,05$).

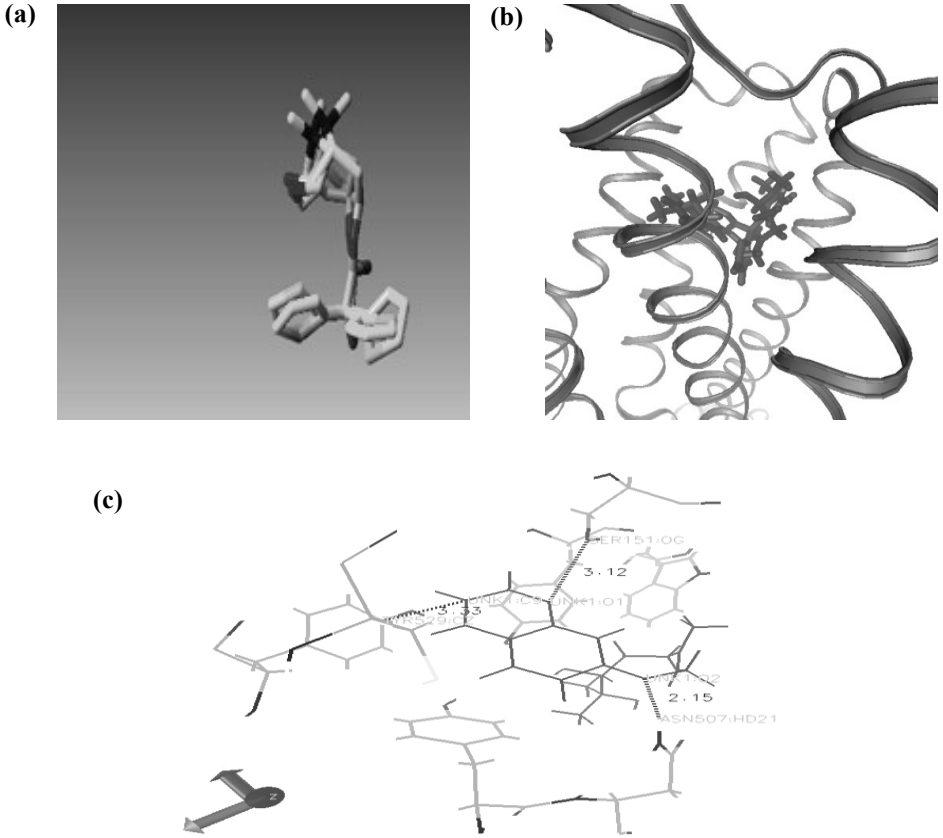


Figure 3 Docking analysis of marmin on ACh M_3 receptors. (a) The results of the validation of the native ligand docking (tiotropium) on the ACh M_3 receptors. (b) Visualization using VMD software. (c) Position of marmin on ACh M_3 receptors.

The decrease of the pD_2 value indicates that marmin had antagonistic activity on the ACh M_3 receptors. Furthermore, a Schild-plot analysis was used to determine the type of antagonism. The result shows that the Schild-plot equation was $y = 0.853x + 1.728$. The slope of the equation was 0.853, close to a value of 1.00. This means that antagonism activity of marmin on the AChM$_3$ receptors was competitive. The marmin pA_2 value as a competitive antagonist was calculated from the intercept of the Schild-plot. The pA_2 of marmin was 1.728.

To evaluate the interaction strength between marmin and AChM$_3$ receptors as competitive antagonists, molecular docking was performed using the PLANTS program. According to the validation step, the RMSD value obtained was 1.5000 Angstrom (< 2.0000 Angstrom) with a docking score of -115.107 (Figure 4 and Table 1). Based on this result, the docking protocol on the ACh M$_3$ receptors was valid. This docking protocol can be used for other AChM$_3$ ligands.

Based on the binding energy data, the binding of marmin to the AChM$_3$ receptors was relatively weak compared to that of the native ligand (tiotropium), as well as some AChM$_3$ antagonists (darifenacin and oxybutymin). However, the marmin binding was stronger than that of the AChM$_3$ receptor agonists (acetylcholine and metacholine) and the AChM$_3$ antagonists (atropine and ipratropium). Figure 3 is a visualization of the molecular docking of marmin on the AChM$_3$ receptors.

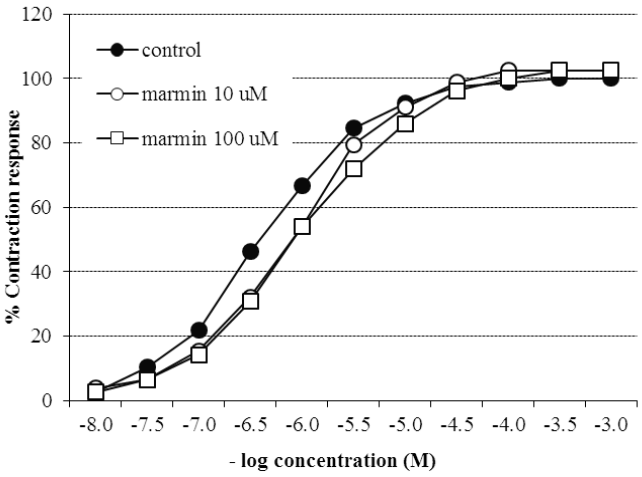
Table 1 Docking score of marmin and other ligands on ACh M$_3$ receptors.

Ligand		**Docking Score on ACh M$_3$ Receptors**
Acetylcholine	ACh M$_3$ agonist	-62.106
Metacholine	ACh M$_3$ agonist	-66.932
Marmin	Test compound	-102.086
Tiotropium	Native ligand	-115.107
Atropine	ACh M$_3$ antagonist	-93.866
Darifenasin	ACh M$_3$ antagonist	-113.465
Oxybutimin	ACh M$_3$ antagonist	-124.584
Ipratropium	ACh M$_3$ antagonist	-91.629

The reversibility assay of marmin is presented in Figure 4. The pD$_2$ value of metacholine decreased significantly. This indicates that the interaction of marmin with the AChM$_3$ receptors could not be dissociated completely by washing for 30 minutes. Nevertheless, the maximal contraction response of the ileum smooth muscle during the reversibility assay could still be achieved. Overall, it can be concluded that by washing for 30 minutes, the binding with the AChM$_3$ receptors was still irreversible.

In previous studies, it was found that an alcoholic extract of *Aegle marmelos* Corr. leaves showed an antagonistic effect on the contraction of isolated ileum induced by histamine. The antagonism doses were 1 and 2 mg/mL [2]. The possible mechanism of the spasmolytic effect is related to inhibition of the activation of the ACh M$_3$ receptors.

(a)



(b)

Groups	pD_2	E_{max}(%)
Control	6.39 ± 0.06	100 ± 0.00
marmin 10 µM	6.12 ± 0.12*	100 ± 0.00
marmin 100 µM	6.05 ± 0.06*	100 ± 0.00

* Significant difference ($P < 0.05$) compared to control.

Figure 4 Reversibility assay: (a) Concentration-response curves to metacholine in guinea-pig ileal smooth muscle after treatment with marmin at concentrations of 10 and 100 µM (data represent n = 5-10, mean ± SEM). (b) pD_2 and Emax values of metacholine (n = 5, mean ± SEM).

The agonist used in this study was methacholine. Methacholine can be attached to the ileum and $AChM_3$ receptors will trigger the ileal smooth muscle to contract. $AChM_3$ is a G protein-linked type receptor. When an agonist binds to this receptor there will be a release of Ca^{2+} from the calcium store. This mechanism is mediated by phospholipase C (PLC). The release of Ca^{2+} from the calcium store results in an increase in intracellular Ca^{2+} levels. Methacholine as $AChM_3$ receptor agonist binds to G proteins and subsequently activates the PLC pathway. Furthermore, the PLC will catalyze the hydrolysis reaction of fosfoinositol 4,5- diphosphate (PIP_2), inositol 1,4,5-triphosphate form (IP_3), and diacyl glycerol (DAG). IP_3 activates IP_3 receptors on the surface of the endoplasmic reticulum. This activation will open transient receptor potential channels (TRPC) and result in the release of Ca^{2+} from the calcium store, after which an increase of the intracellular Ca^{2+} concentration occurs. Increased levels of intracellular Ca^{2+} can activate voltage-dependent Ca^{2+} channels in the cell membrane surface [8,9]. Activation of calcium ion channels on the cell

membrane surface results in an influx of extracellular Ca^{2+}. This overall pathway increases the levels of intracellular Ca^{2+}, which could induce smooth muscle contraction [10].

Marmin at doses of 10 and 100 μM inhibited a smooth muscle contraction response of isolated guinea pig ileum induced by a concentration series of methacholine. This was shown by a dose-dependent shift to the right in the response curve of the isolated ileal smooth muscle contraction. The pD_2 value of methacholine shifted significantly from 6.39 to 5.46. The pA_2 value was 1.728 (marmin dose 100 uM). At a dose of 10 uM marmin, the pD_2 shifted from 6.39 to 6.08, however, this shift is not significant. This shows that marmin at a dose of 10 uM is not effective as an antagonist of $AChM_3$, whereas the concentration of 100 uM was effective.

Although marmin acts as an antagonist of $AChM_3$, the maximum response (E_{max}) can still be achieved by administration of methacholine agonist with higher concentrations. Marmin can occupy the same binding site as methacholine on $AChM_3$ receptors. Based on these results, it follows that the antagonistic action of the $AChM_3$ receptors on marmin was competitive. This was confirmed by the results of the Schild-plot analysis. The Schild-slope value approached to a value of one (0.853). With the Schild-plot of the curve, the pA_2 value was also obtained (1.728). This shows that the affinity of marmin to the $AchM_3$ receptors was relatively weak. To further study the bond strength of marmin to the $AChM_3$ receptors, a docking simulation using the PLANTS program was then performed.

The validation values (RMSD) were obtained at 1.5000 Angstrom, with a docking score of -115.107. If the native ligand RMSD value is less than 2.0000 Angstrom, the docking system is valid. The docking score of marmin to $AChM_3$ receptor obtained was -102.086. The docking scores were below the score of the native ligand. These results indicate that marmin's affinity with the $AChM_3$ receptors was weaker than with tiotropium.

To reveal the bond strength between marmin and the $AChM_3$ receptors more clearly, the docking scores were compared with those of some $AChM_3$ antagonists. The $AChM_3$ antagonists used in this study were atropine, ipratropium (tropan derivative), darifenacin, and oxybutimin. Atropine as an $AChM_3$ antagonist is often used to induce mydriasis on eye muscles. Ipratropium as an ACh M_3 antagonist is often used as a bronchodilator, as is tiotropium. Darifenacin and oxybutimin are used to relax bladder spasms (overactive bladder). Marmin's docking scores (-102.086) were higher when compared with atropine (-93.866) and ipratropium (-91.629). However, when

compared with the scores of darifenacin (-113.465) and oxybutimin (-124.584), marmin showed weaker affinity.

4 Conclusion

Based on these results, it can be concluded that marmin has activity as a competitive antagonist on $AChM_3$ receptors. Based on our *in silico* study, marmin is bound to $AChM_3$ receptors, however, the binding is weaker than that of native ligand (tiotropium) and stronger than that of its agonists and antagonists.

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